

DNA CODING FOR PROTEIN WHICH CONFERS ON BACTERIUM
ESCHERICHIA COLI RESISTANCE TO L-HOMOSERINE, AND
METHOD FOR PRODUCING L-AMINO ACIDS

Technical Field

5 The present invention relates to a method for
producing an amino acid, especially for a method for
producing L-homoserine, L-alanine, L-isoleucine, L-
valine, or L-threonine using a bacterium belonging to
the genus *Escherichia*.

10 Background Art

 The present inventors obtained, with respect to
E. coli K-12, a mutant having mutation, *thrR*, (herein
referred to as *rhtA23*) that is concerned in high
concentrations of threonine (>40 mg/ml) or homoserine
15 (>5 mg/ml) in a minimal medium (Astaurova, O. B. et
al., Appl. Bioch. and Microbiol., 21, 611-616
(1985)). On the basis of *rhtA23* mutation an improved
threonine-producing strain (SU patent No. 974817),
homoserine- and glutamic acid-producing strains
20 (Astaurova et al., Appl. Bioch. And Microbiol., 27,
556-561 (1991)) were obtained.

 Furthermore, the present inventors has revealed
that the *rhtA* gene exists at 18 min on *E. coli*

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chromosome and that the *rhtA* gene is identical to
ORF1 between *pexB* and *ompX* genes. The unit
expressing a protein encoded by the ORF1 has been
designated as *rhtA* (*rht*: resistance to homoserine and
5 threonine) gene. The *rhtA* gene includes a 5'-
noncoding region including SD sequence, ORF1 and a
terminator. Also, the present inventors have found
that a wild type *rhtA* gene participates in resistance
to threonine and homoserine if cloned in a multicopy
10 state and that enhancement of expression of the *rhtA*
gene improves amino acid productivity of a bacterium
belonging to the genus *Escherichia* having an ability
to produce L-lysine, L-valine or L-threonine
(ABSTRACTS of 17th International Congress of
15 Biochemistry and Molecular Biology in conjugation
with 1997 Annual Meeting of the American Society for
Biochemistry and Molecular Biology, San Francisco,
California August 24-29, 1997, abstract No. 457).

It is found that at least two different genes
20 which impart homoserine resistance in a multicopy
state exist in *E. coli* during cloning of the *rhtA*
gene. One of the genes is the *rhtA* gene, however the
other gene has not been elucidated.

Disclosure of the Invention

25 An object of the present invention is to provide

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a novel gene participating in resistance to homoserine, and a method for producing an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine with a high yield.

5 The inventors have found that a region at 86 min on *E. coli* chromosome, when cloned by a multicopy vector, impart resistance to L-homoserine to cells of *E. coli*, and that when the region is amplified, the amino acid productivity of *E. coli* can be improved
10 like the *rhtA* gene. On the basis of these findings, the present invention have completed.

 Thus, the present invention provides:

(1) a DNA coding for a protein as defined in the following (A) or (B):

15 (A) a protein which comprises an amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing; or

 (B) a protein which comprises an amino acid sequence including deletion, substitution, insertion
20 or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing, and which has an activity of making a bacterium having the protein L-homoserine-resistant,
 (2) the DNA according to (1), which is a DNA as
25 defined in the following (a) or (b):

 (a) a DNA which comprises a nucleotide sequence

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corresponding to the nucleotide numbers of 557 to 1171 of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing; or

- (b) a DNA which hybridizes with the nucleotide sequence corresponding to the nucleotide numbers of 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for the protein having the activity of making the bacterium having the protein L-homoserine-resistant,
- (3) a bacterium belonging to the genus *Escherichia*, wherein L-homoserine resistance of the bacterium is enhanced by amplifying a copy number of the DNA of (1) in a cell of the bacterium,
- (4) the bacterium of (3), wherein the DNA of (1) is carried on a multicopy vector in the cell of the bacterium,
- (5) the bacterium of (3), wherein the DNA of (1) is carried on a transposon in the cell of the bacterium,
- (6) a method for producing an amino acid, comprising the steps of cultivating the bacterium of any of (3) to (5), which has an ability to produce the amino acid, in a culture medium to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium, and
- (7) the method of (6), wherein the amino acid is at least one selected from the group consisting of L-

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homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

5 The DNA of the present invention may be referred to as "*rhtB* gene", a protein coded by the *rhtB* gene may be referred to as "RhtB protein", an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of making a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as "Rh activity", and a structural gene encoding the RhtB protein in the *rhtB* gene may be referred to as "*rhtB* structural gene". The term "enhancing the Rh activity" means imparting resistance to homoserine to a bacterium or enhance the resistance by means of increasing the number of molecules of the RhtB protein, increasing a specific activity of the RhtB protein, or desensitizing negative regulation against the expression or the activity of the RhtB protein or the like. The terms "DNA coding for a protein" mean a DNA of which one of strands codes for the protein when the DNA is double-stranded. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild type strain thereof can not grow, usually at 10 mg/ml. The ability to produce an amino acid means a property that a

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bacterium produces and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

According to the present invention, resistance to homoserine of a high concentration can be imparted to a bacterium belonging to the genus *Escherichia*. A bacterium belonging to the genus *Escherichia*, which has increased resistance homoserine and an ability to accumulate an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine or L-threonine in a medium with a high yield.

The present invention will be explained in detail below.

<1> DNA of the present invention

The DNA of the present invention coding for a protein having the Rh activity and having an amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing. Specifically, the DNA of the present invention may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing.

The DNA of the present invention includes a DNA fragment encoding the RhtB protein conferring bacterium *Escherichia coli* resistance to homoserine, which includes the regulatory elements of the *rhtB*

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gene and the structural part of *rhtB* gene, having the nucleotide sequence shown in SEQ ID NO: 1.

5 The nucleotide sequence shown in SEQ ID NO: 1 corresponds to a part of sequence complement to the sequence of GenBank accession number M87049. SEQ ID NO: 1 includes f138 (nucleotide numbers 61959-61543 of GenBank accession number M87049) which is a known but function-unknown ORF (open reading frame) present at 86 min on *E. coli* chromosome, and 5'-flanking and 10 3'-flanking regions thereof. The f138, which had only 160 nucleotides in the 5'-flanking region, could not impart the resistance to homoserine. No termination codon is present between the 62160 and 61959 of M87049 (upstream the ORF f138). Hence, the 15 coding region is 201 bp longer. Thus the RhtB protein and the *rhtB* gene coding for the protein are novel.

20 The *rhtB* gene may be obtained, for example, by infecting Mucts lysogenic strain of *E. coli* using a lysate of a lysogenic strain of *E. coli* such as K12 or W3110 according to the method in which mini-Mu d5005 phagemid is used (Groisman, E. A., et al., J. Bacteriol., 168, 357-364 (1986)), and isolating plasmid DNAs from colonies growing on a minimal 25 medium containing kanamycin (40 µg/ml) and L-homoserine (10 mg/ml). As illustrated in the Example

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described below, the *rhtB* gene was mapped at 86 min on the chromosome of *E. coli*. Therefore, the DNA fragment including the *rhtB* gene may be obtained from the chromosome of *E. coli* by colony hybridization or PCR (polymerase chain reaction, refer to White, T.J. et al, Trends Genet. 5, 185(1989)) using oligonucleotide(s) which has a sequence corresponding to the region near the portion of 86 min on the chromosome of *E. coli*. Alternatively, the oligonucleotide may be designed according to the nucleotide sequence shown in SEQ ID NO: 1. By using oligonucleotides having nucleotide sequences corresponding to a upstream region from the nucleotide number 557 and a downstream region from the nucleotide number 1171 in SEQ ID NO: 1 as the primers for PCR, the entire coding region can be amplified.

Synthesis of the oligonucleotides can be performed by an ordinary method such as a phosphoamidite method (see Tetrahedron Letters, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.), using Taq

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The DNA coding for the RhtB protein of the present invention may code for RhtB protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rh activity of RhtB protein encoded thereby is not deteriorated. The DNA, which codes for the substantially same protein as the RhtB protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site involve deletion, substitution, insertion or addition. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA coding for the RhtB protein *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia* harboring a DNA coding for the RhtB protein with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid usually used

for the mutation treatment.

5 The DNA, which codes for substantially the same
protein as the RhtB protein, can be obtained by
expressing a DNA subjected to in vitro mutation
treatment as described above in multicopy in an
appropriate cell, investigating the resistance to
homoserine, and selecting the DNA which increase the
resistance. Also, it is generally known that an
amino acid sequence of a protein and a nucleotide
10 sequence coding for it may be slightly different
between species, strains, mutants or variants, and
therefore the DNA, which codes for substantially the
same protein, can be obtained from L-homoserine-
resistant species, strains, mutants and variants
15 belonging to the genus *Escherichia*. Specifically,
the DNA, which codes for substantially the same
protein as the RhtB protein, can be obtained by
isolating a DNA which hybridizes with DNA having, for
example, a nucleotide sequence of the nucleotide
20 numbers 557 to 1171 of the nucleotide sequence shown
in SEQ ID NO: 1 in Sequence Listing under stringent
conditions, and which codes for a protein having the
Rh activity, from a bacterium belonging to the genus
Escherichia which is subjected to mutation treatment,
25 or a spontaneous mutant or a variant of a bacterium
belonging to the genus *Escherichia*. The term

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5 "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized.

10 <2> Bacterium belonging to the genus *Escherichia* of the present invention

15 The bacterium belonging the genus *Escherichia* of the present invention is a bacterium belonging to the genus *Escherichia* of which the Rh activity is enhanced. A bacterium belonging to the genus *Escherichia* is exemplified by *Escherichia coli*. The Rh activity can be enhanced by, for example, amplification of the copy number of the *rhtB* structural gene in a cell, or transformation of a bacterium belonging to the genus *Escherichia* with a recombinant DNA in which a DNA fragment including the *rhtB* structural gene encoding the RhtB protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*. The Rh activity can be also enhanced by

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substitution of the promoter sequence of the *rhtB* gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus *Escherichia*.

5 The amplification of the copy number of the *rhtB* structural gene in a cell can be performed by introduction of a multicopy vector which carries the *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*. Specifically,
10 the copy number can be increased by introduction of a plasmid, a phage or a transposon (Berg, D.E. and Berg, C. M., Bio/Technol., 1, 417 (1983)) which carries the *rhtB* structural gene into a cell of a bacterium belonging to the genus *Escherichia*.

15 The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as λ 1059, λ BF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

20 The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. M. Morrison (Methods in Enzymology 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium
25 chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and the

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like.

If the Rh activity is enhanced in an amino acid-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus *Escherichia* which is to be the Rh activity is enhanced, strains which have abilities to produce desired amino acids are used. Besides, an ability to produce an amino acid may be imparted to a bacterium in which the Rh activity is enhanced. Examples of amino acid-producing bacteria belonging to the genus *Escherichia* are described below.

(1) L-threonine-producing bacteria

The L-threonine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain MG442 (Guayatiner et al., Genetika (in Russian), 14, 947-956 (1978)).

(2) L-homoserine-producing bacteria

The L-homoserine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain NZ10 (*thrB*). This strain was derived from the known strain C600 (*thrB*, *leuB*) (Appleyard R.K., Genetics, 39, 440-452 (1954)) as Leu⁺ revertant.

On the basis of the *rhtB* DNA fragment, new amino acid-producing strains *E. coli* NZ10/pAL4,pRhtB; *E. coli* MG422/pVIC40,pRhtB; and *E. coli* MG442/pRhtB were

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Cultural features:

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mm in diameter having a smooth surface, homogeneous structure, pastelike consistency, readily emulsifiable.

Minimal agar-doped medium M9. After 40 to 48 hours of growth at 37°C, forms colonies 0.5 to 1.5 mm in diameter, which are colored greyish-white, semitransparent, slightly convex, with a lustrous surface.

Growth in a beef-extract broth. After 24-hour growth at 37°C, exhibits strong uniform cloudiness, has a characteristic odor.

Physiological and biochemical features:

Grows upon thrust inoculation in a beef-extract agar. Exhibits good growth throughout the inoculated area.

The microorganism proves to be a facultative anaerobe.

It does not liquefy gelatin.

Features a good growth on milk, accompanied by milk coagulation.

Does not produce indole.

Temperature conditions. Grows on beef-extract broth at 20-42°C, an optimum temperature lying within 33-37°C.

pH value of culture medium. Grows on liquid media having the pH value from 6 to 8, an optimum value being 7.2.

Carbon sources. Exhibits good growth on glucose,

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fructose, lactose, mannose, galactose, xylose,
glycerol, and mannitol to produce an acid and gas.
Nitrogen sources. Assimilates nitrogen in the form
of ammonium, nitric acid salts, as well as from some
5 organic compounds.

Resistant to ampicillin, kanamycin and L-homoserine.
L-Threonine is used as a growth factor.

Content of plasmids. The cells contain multicopy
hybrid plasmid pAL4 ensuring resistance to ampicillin
10 and carrying the gene *thrA* of the threonine operon,
which codes for aspartate kinase-homoserine
dehydrogenase I responsible for the increased
homoserine biosynthesis. Besides, the cells contain
a multicopy hybrid plasmid pRhtB ensuring resistance
15 to kanamycin and carrying the *rhtB* gene which confers
resistance to homoserine (10 mg/l).

The strain *E. coli* MG442/pRhtB (VKPM B-7659) has
the same cultural-morphological and biochemical
features as the strain NZ10/pAL4,pRhtB except for L-
20 isoleucine is used as a growth factor instead of L-
threonine. However, the strain can grow slowly
without isoleucine. Besides, the cells of the strain
contain only one multicopy hybrid plasmid pRhtB
ensuring resistance to kanamycin and carrying the
25 *rhtB* gene which confers resistance to homoserine (10
mg/l).

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The strain *E. coli* MG442/pVIC40,pRhtB (VKPM B-7660) has the same cultural-morphological and biochemical features as the strain NZ10/pAL4,pRhtB except for L-isoleucine is used as a growth factor instead of L-threonine. However, the strain can grow slowly without isoleucine. The cells of the strain contain multicopy hybrid plasmid pVIC40 ensuring resistance to streptomycin and carrying the genes of the threonine operon. Besides, they contain multicopy hybrid plasmid pRhtB ensuring resistance to kanamycin and carrying the *rhtB* gene which confers resistance to homoserine (10 mg/l).

<3> Method for producing an amino acid

An amino acid can be efficiently produced by cultivating the bacterium in which the Rh activity is enhanced by amplifying a copy number of the *rhtB* gene as described above, and which has an ability to produce the amino acid, in a culture medium, producing and accumulating the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino

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acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be
5 either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include
10 various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as
15 ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyte and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate,
20 manganese sulfate, calcium carbonate are used.

The cultivation is preferably culture under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C.
25 The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium

carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of the target amino acid in the medium.

Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

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Brief Explanation of Drawing

Fig. 1 shows cloning, identification and inactivation of the *rhtB* gene.

Fig. 2 shows the amino acid sequence of the RhtB protein.

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Examples

The present invention will be more concretely explained below with reference to Examples. In the Examples, an amino acid is of L-configuration unless otherwise noted.

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Example 1: Obtaining of *rhtB* DNA fragment

(1) Cloning of *rhtB* gene into mini-Mu phagemid

The wild-type *rhtB* gene was cloned *in vivo* using mini-Mu d5005 phagemid (Groisman, E. A., et al., J.

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Bacteriol., 168, 357-364 (1986)). MuCts62 lysogen of the strain MG442 was used as a donor. Freshly prepared lysates were used to infect a Mucts lysogenic derivative of a strain VKPM B-513 (Hfr K10 metB). The cells were plated on M9 glucose minimal medium with methionine (50 µg/ml), kanamycin (40 µg/ml) and homoserine (10 mg/ml). Colonies which appeared after 48 hr were picked and isolated. Plasmid DNA was isolated and used to transform the strain VKPM B-513 by standard techniques. Transformants were selected on L-broth agar plates with kanamycin as above. Plasmid DNA was isolated from those which were resistant to homoserine, and analyzed by restriction mapping of the structure of the inserted fragments. It appeared that two types of inserts belonging to different chromosome regions had been cloned from the donor. Thus, at least two different genes that is in multicopy and imparts resistance to homoserine exist in *E. coli*. One of the two type of inserts is the *rhtA* gene which has already reported (ABSTRACTS of 17th International Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997). Among the other of the two types of inserts, a fragment of a

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minimum length which imparts the resistance to
homoserine is of 0.8 kb (Fig. 1).

(2) Identification of *rhtB* gene

5 The insert fragment was sequenced by the dideoxy
chain termination method of Sanger. Both DNA strands
were sequenced in their entirety and all junctions
were overlapped. The sequencing showed that the
insert fragment included f138 (nucleotide numbers
61543 to 61959 of GenBank accession number M87049)
10 which was a known but function-unknown ORF (open
reading frame) present at 86 min of *E. coli*
chromosome and 201 bp of an upstream region thereof
(downstream region in the sequence of M87049). The
f138 which had only 160 nucleotides in the 5'-
15 flanking region could not impart the resistance to
homoserine. No termination codon is present upstream
the ORF f138 between 62160 and 61959 nucleotides of
M87049. Furthermore, one ATG following a sequence
predicted as a ribosome binding site is present in
20 the sequence. The larger ORF (nucleotide numbers
62160 to 61546) is designated as *rhtB* gene. The RhtB
protein deduced from the gene is highly hydrophobic
and contains 5 possible transmembrane segments.

Example 2: Production of homoserine-producing strain

25 Strain NZ10 of *E. coli* was transformed by a

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plasmid pAL4 which was a pBR322 vector into which the
thrA gene coding for aspartokinase-homoserine
dehydrogenase I was inserted, to obtain the strains
NZ10/pAL4. The strain NZ10 is a *leuB*⁺-reverted mutant
5 (*thrB*) obtained from the *E. coli* strain C600 (*thrB*,
leuB) (Appleyard, Genetics, 39, 440-452 (1954)).

The *rhtB* gene was inserted to a plasmid pUK21
which is a known plasmid pUC19 in which a kanamycin
resistance gene substituted for an ampicillin
10 resistance gene (Vieira, J. and Messing, J., Gene,
100, 189-194 (1991)), to obtain pRhtB.

The strain NZ10/pAL4 was transformed with pUK21
or pRhtB to obtain strains NZ10/pAL4,pUK21 and
NZ10/pAL4,pRhtB.

15 The thus obtained transformants were each
cultivated at 37°C for 18 hours in a nutrient broth
with 50 mg/l kanamycin and 100 mg/l ampicillin, and
0.3 ml of the obtained culture was inoculated into 3
ml of a fermentation medium having the following
20 composition and containing 50 mg/l kanamycin and 100
mg/l ampicillin, in a 20 x 200 mm test tube, and
cultivated at 37°C for 46 hours with a rotary shaker.
After the cultivation, an accumulated amount of
homoserine in the medium and an absorbance at 560 nm
25 of the medium were determined by known methods.

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Fermentation medium composition (g/L)

	Glucose	80
	$(\text{NH}_4)_2\text{SO}_4$	22
	K_2HPO_4	2
5	NaCl	0.8
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.8
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.02
	$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	0.02
	Thiamine hydrochloride	0.0002
10	Yeast Extract	1.0
	CaCO_3	30
	(CaCO ₃ was separately sterilized.)	

The results are shown in Table 1. As shown in Table 1, the strain NZ10/pAL4,pRhtB accumulated homoserine in a larger amount than the strains NZ10/pAL4 and NZ10/pAL4,pUK21 in which the *rhtB* gene was not enhanced.

Table 1

Strain	OD ₅₆₀	Accumulated amount of homoserine (g/L)
NZ10/pAL4	16.4	3.1
NZ10/pAL4,pUK21	14.3	3.3
NZ10/pAL4,pRhtB	15.6	6.4

Example 3: Production of alanine, valine and isoleucine
with pRhtB-introduced strain

E. coli strain MG442 is a known strain (Gusyatiner, et al., 1978, Genetika (in Russian), 14:947-956).

5 The strain MG442 was transformed with the plasmids pUK21 and pRhtB to obtain strains MG442/pUK21 and MG442/pRhtB.

10 The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 3 and containing 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 40 hours with a rotary shaker. After the cultivation, accumulated amounts of alanine, valine and isoleucine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

15 The results are shown in Table 2. As shown in Table 2, the strain MG442/pRhtB accumulated each of alanine, valine and isoleucine in a larger amount than the strain MG442/pUK21 in which the *rhtB* gene was not enhanced.

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Table 2

Strain	OD ₅₆₀	Accumulated amount (g/L)		
		Alanine	Valine	Isoleucine
MG442/pUK21	13.4	0.2	0.2	0.3
MG442/pRhtB	13.7	0.7	0.5	0.5

Example 4: Production of threonine-producing strain

The strain MG442 (Example 3) was transformed by introducing a known plasmid pVIC40 (U.S. Patent No. 5,175,107 (1992)) by an ordinary transformation method. Transformants were selected on LB agar plates containing 0.1 mg/ml streptomycin. Thus a novel strain MG422/pVIC40 was obtained.

The strain MG442/pVIC40 was transformed with pUK21 or pRhtB to obtain strains MG442/pVIC40,pUK21 and MG442/pVIC40,pRhtB.

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin and 100 mg/l streptomycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 3 and containing 50 mg/l kanamycin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by

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Strain	OD ₅₆₀	Accumulated amount of threonine (g/L)
MG442/pVIC40	17	13.6
MG442/pVIC40, pUK21	16.3	12.9
MG442/pVIC40, pRhtB	15.2	16.3

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plasmid (Fig. 1). To introduce the obtained
insertionally inactivated *rhtB* gene into the chromosome
of the *E. coli* strain N99 (the streptomycin-resistant
derivative of the known strain W3350 (Campbell,
5 Virology, 14, 22-33, 1961)), the method of Parker and
Marinus was used (Parker, B. and Marinus, M. G., Gene,
73, 531-535, 1988). The substitution of the wild type
allele for the inactivated one was proved by phage P1
transduction and by Southern hybridization (Southern,
10 E. M., J. Mol. Biol., 98, 503-517, 1975).

Then the susceptibility of the thus obtained *E.*
coli strain N99 *rhtB::cat*, of the initial strain N99
(*rhtB*⁻) and of its derivative transformed with pRhtB
plasmid, N99/pRhtB, to some amino acids and amino acid
15 analogues was tested. Overnight cultures of the
strains grown in M9 minimal medium at 37°C with a
rotary shaker (10⁹ cfu/ml) were diluted 1:100 and grown
for 5 hours under the same conditions. Then the log
phase cultures thus obtained were diluted and about 10⁴
20 of alive cells were applied to well-dried test plates
with M9 agar containing doubling increments of amino
acids or analogues. The minimum inhibitory
concentration (MIC) of these compounds were examined
after 40-46 h cultivation. The results are shown in
25 Table 4.

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Table 4

Substrate	MIC ($\mu\text{g/ml}$)		
	N99(<i>rhtB</i> ⁺)	N99/pRhtB	N99 <i>rhtB::cat</i>
1. L-homoserine	250	30000	125
2. L-threonine	30000	50000	30000
3. L-serine	5000	10000	5000
4. L-valine	0.5	1	0.5
5. AHVA	50	2000	25
6. AEC	10	25	10
7. 4-aza-DL-leucine	40	100	40

It follows from the Table 4 that multiple copies of *rhtB* besides homoserine conferred upon cells increased resistance to threonine, serine, valine, α -amino- β -hydroxyvaleric-acid (AHVA), S-(2-aminoethyl)-L-cysteine (AEC), and 4-aza-DL-leucine. The inactivation of the *rhtB* gene, on the contrary, increased the cell sensitivity to homoserine and AHVA. These results in conjunction with the data on homology of the RhtB protein to LysE lysine efflux transporter of *Corynebacterium glutamicum* (Vrljic et al., Mol. Microbiol., 22, 815-826, 1996) indicate the analogues function for the *rhtB* gene product. The presumed efflux transporters, RhtB, has specificity to several substrates (amino acids), or may show non-specific effects as a result of amplification.